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# Isolation and Identification of Methylotrophic Bacteria Producing Methanol Dehydrogenase from Human Feet and Mouth

**DIANA ELIZABETH WATURANGI*, CATHERINE DELANY NICHOLAS, CINDY OKTAVIA SUSANTO, MAGGY THENAWIJAYA SUHARTONO**

*Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia,*
*Jalan Jenderal Sudirman 51, Jakarta Selatan 12930, Indonesia*

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**The human feet and mouth are known as sources of methylated sulfides, which are produced by other microflora. Methylated sulfides could be oxidized by methylotrophic bacteria, which may result in odor reduction in human feet and mouth. In this study, we collected a total of 21 isolates from human feet, and 37 isolates from human mouth. These isolates were identified with biochemical test such as oxidase and catalase test and Gram staining assay. The presence of *mxaF* gene of methanol dehydrogenase was detected by PCR using specific primers. However, the result showed that most of the isolates did not possess *mxaF* gene. Hence, the methanol dehydrogenase (MDH) activity was also determined. From the total 21 isolates obtained from the feet, only 15 of them showed MDH activity whereas 23 isolates from the total 37 isolates obtained from teeth and tongue region also showed MDH activity. Isolate K25-3 (74.444 U/ml), K33-6 (79.815 U/ml), and K43-5 (69.259 U/ml) from human feet and M41L3 (135.926 U/ml), M27G2 (85.556 U/ml), and M51G1 (103.333 U/ml) from human mouth showed the highest total enzyme activity. Isolates with the highest total activity could be used for further studies such as purification of the enzyme and isolates characterization.**

Key words: methylotrophic bacteria; methanol dehydrogenase

## INTRODUCTION

Methylotrophic bacteria are aerobic bacteria that utilize one-carbon compounds, such as methane, methanol, and methylated compounds containing sulfur, as sources of carbon and energy (Hanson & Hanson 1996). Methylotrophic bacteria are found in a variety of habitats such as leaf, polluted water, air, soil (Pasamba *et al.* 2007), drinking water (Gallego *et al.* 2005), vehicular soot (Jang & Lee 2008), and rice (Madhaiyan *et al.* 2007). Interestingly, methylotrophic bacteria are also found as a normal part of microflora in human mouth and feet (Anesti *et al.* 2004; Anesti *et al*. 2005).

The human mouth and feet are sources of volatile one carbon compounds such as methanethiol and dimethylsulphide. These compounds are produced by diverse bacteria colonizing the human mouth and foot habitat (Goldberg *et al.* 1997; Anesti *et al*. 2004; Anesti *et al.* 2005). Thus, methylotrophic bacteria occur in that habitat and utilize volatile one carbon compounds as their energy source. The enzymes of methylotrophic metabolism play an important role in methylotrophic bacteria's ability to use one carbon compounds as energy source. One of these methylotrophic enzymes is methanol dehydrogenase (MDH).

MDH carries out a key step in methylothropic bacteria since it catalyzes oxidation of methanol to formaldehyde; after which the formaldehyde can further metabolize to formate through some reaction (Chistorserdova *et al.* 2004). MDH exists in an α2β2 tetramer. The larger? α-subunit is 66 kDa and the? β-subunit is only 8.5 kDa. In the active site, there are pyroloquinoline quinon (PQQ) and a $Ca^{2+}$ ion which are bound non-covalently to each of α subunit. They are both key to the action of methanol dehydrogenase (Liu *et al.* 2006).

Genetics of methanol oxidation in *Methylobacterium extorquens* AM1 have been studied. There are at least 17 genes that play a role in this process. Three of these genes encode the structural protein of a methanol oxidation complex. The *mxaF* and *mxaI* encode the large á and small β subunit of MDH. The *mxaG* encodes the primary electron acceptor for MDH (McDonald & Murel 1997).

Although there are some studies about the methylotrophic bacteria, there are still quite a few. Moreover, these studies were done in subtropical areas. Regarding this issue, it is important to investigate the methylotrophic bacteria occurring in tropical area. We now report the isolation and identification of methylotrophic bacteria in human mouth and feet from a tropical region. The activity of methanol dehydrogenase was also determined.

## MATERIALS AND METHODS

**Methylotrophic Bacteria Isolation from Human Feet and Mouth.** Isolates were collected from 55 subjects (age 20-22) lived in Jakarta, Indonesia. Samples were obtained from soles and toe clefts by using sterile cotton buds soaked with 0.85% NaCl. Samples were also obtained from human mouth within dental region and tongue by

*Corresponding author. Phone: +62-21-5703306 ext. 330, Fax: +62-21-5719060, E-mail: diana.waturangi@atmajaya.ac.id

swabbing with sterile cotton bud. The sample obtained on cotton buds was streaked directly onto minimal media (bacteriological agar) supplemented with 1% (v/v) methanol (Riupassa & Suwanto 2004). After that, the media were incubated at 28 °C for approximately 1 week. Methylotrophic bacteria were then isolated and stored in minimal media supplemented with 1% (v/v) methanol and Luria agar media supplemented with 0.01% (w/v) cycloheximide (Sigma-Aldrich,USA) to reduce fungi contamination (Riupassa & Suwanto 2004).

**Methylotrophic Bacteria Identification.** Biochemical assays used to identify the isolates were catalase test and oxidase test. Gram staining was also performed to differentiate the isolates.

**Extraction of Genomic DNA.** Cultures were enriched in Luria Agar medium at 28 °C for 2 days and genomic DNA was extracted using Cetyltrimethylammonium bromide (CTAB) method (Marchesi *et al*. 1998).

**Detection of *mxaF* Gene by PCR.** Amplification of *mxaF* gene was achieved by amplification with specific primer for *mxaF* gene. Primer used in this study are primer f1003 (5'-GCGGCACCAACTGGGCTGGT) and r1561 (5'-GGGCAGCATGAAGGGCTCCC), according to Mcdonald and Murrel (1997) and Anesti *et al*. (2005). PCR amplification was performed in 25 µl reaction mixture with Gene PCR System 2700 (Applied biosystems). Reactant used for amplification was GoTaq® Green Master Mix. The PCR conditions were 30 cycles of 92 °C for 1 minute, 55 °C for 1 minute, and 72 °C for 1 minute. The presence of single band of expected size (550 bp) was confirmed by 0.8% agarose gel electrophoresis.

**Cultivation of Bacteria for Enzyme Assay.** Bacteria cultivation method was based on a research by Liu *et al.* (2006). The media was modified with Luria broth [1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl] and cultivated at 28 °C, 110 rpm for 66 hours.

**Enzyme Extraction.** Bacteria isolates were harvested by centrifugation (Sorvall Legend™ RT) at 9.000 × g for 45 minutes. The pellet was suspended in distilled water (w/v= 1:4). Intracellular enzyme and methanol dehydrogenase from periplasm space were obtained by breaking down the membrane of bacteria. The suspension was treated for 2 minutes with an ultrasonic device (Biologics Inc., ultrasonic homogenizer, model 150 V/T), output 50, using 65% pulses. The lysate was then centrifuged at 9.000 × g (Sorvall Legend™ RT) for 60 minutes to separate the cell from other solute. The resulting supernatant was mixed with 100 mM 4-morpholine ethanesulfonic acid (Mes) (Sigma-Aldrich, USA) (v/v= 3:1) to a final buffer concentration of 25 mM (Liu *et al.* 2006). Crude extract was stored in freezer (- 20 °C) for further analysis.

**Enzyme Assay.** The assay mixture contained 100 mM Tris-HCl buffer pH 8, 15 mM $NH_4Cl$, 10 mM $CH_3OH$ as substrate, 50 µM 6-dichloroindophenolate (DCIP) (Sigma-Aldrich, Austria), 0.33 mM Phenazine methosulfaten (PMes) (Sigma-aldrich, Germany) and the crude extract (75-150 µl). The reaction was initiated by the addition of PMes and methanol dehydrogenase activity was measured by the decrease of DCIP absorbance at 600 nm within 1 minute (Liu *et al*. 2006) at pH 8.0 and room temperature (25 °C). The assay mixture without enzyme served as the control. One unit of enzyme activity was defined as the amount that catalyzed the reduction of 1 µmol DCIP per minute. Protein concentrations were determined by the Bradford method with Bovine Serum albumin (BSA) as the standard (Bradford 1976).

## RESULTS

**Methylotrophic Bacteria Isolation from Human Feet and Mouth.** From 55 human subjects (age 20-22 years) lived in Jakarta, Indonesia, a total of 58 isolates collected. 21 of them were obtained from human feet, and 37 of them from human mouth.

**Methylotrophic Bacteria Identification.** The colors of the isolates vary between pink, beige, orange, and yellow. Various morphology and different results of oxidase and catalase test were also shown among the isolates (Table 1 & 2).

Table 1. The biochemical properties and characteristics of the isolates obtained from human feet

| Isolates | Colony color on luria agar | Morphology | Gram staining | Oxidase test | Catalase test | Detection of *MxaF* gene |
|---|---|---|---|---|---|---|
| K4-1 | Orange | Coccobacil | - | - | + | - |
| K4-2 | Orange | Coccobacil | - | - | + | - |
| K4-3 | Beige | Coccobacil | - | - | + | + |
| K9 | Orange | Coccus | - | - | + | - |
| K12-2 | Beige | Coccus | - | - | + | - |
| K18-1 | Pink | Coccus | - | - | + | - |
| K19-2 | Yellow | Coccus | - | - | + | - |
| K25-1 | Pink | Coccus | - | - | + | - |
| K25-2 | Pink | Coccus | - | - | + | - |
| K25-3 | Pink | Coccus | - | - | + | - |
| K28 | Beige | Coccus | - | - | + | - |
| K32-1 | Pink | Coccus | - | - | + | - |
| K32-2 | Pink | Coccus | - | - | + | - |
| K33-6 | Pink | Coccobacil | - | - | + | - |
| K33-7 | Pink | Bacil | - | - | + | - |
| K41-3 | Beige | Coccus | - | - | + | - |
| K42 | Pink | Coccobacil | - | - | + | - |
| K43-1 | Pink | Coccus | - | - | + | - |
| K43-4 | Pink | Coccus | - | - | + | - |
| K43-5 | Beige | Bacil | - | - | + | - |
| K45-2 | Beige | Coccobacil | - | - | + | - |

Table 2. The biochemical properties and characteristics of the isolates obtained from human mouth

| Isolates | Colony color on luria agar | Morphology | Gram staining | Oxidase test | Catalase test | Detection of *MxaF* gene |
|---|---|---|---|---|---|---|
| M2G1 | White | Bacil | + | - | + | - |
| M7L1 | Yellow | Coccus | - | - | + | - |
| M12L1 | Orange | Coccus | - | - | + | - |
| M18G1 | Orange | Bacil | - | + | + | - |
| M22L1 | Pink-Orange | Coccobacil | - | - | + | - |
| M23L1 | White | Bacil | - | + | + | - |
| M23G2 | White | Bacil | - | - | + | - |
| M24G1 | Yellow | Coccus | - | - | + | - |
| M27L1 | Orange | Coccus | - | - | + | - |
| M27L2 | White | Coccus | - | - | + | - |
| M27L3 | White | Coccus | - | - | + | - |
| M27G1 | White | Coccus | - | - | + | - |
| M27G2 | Creamy-white | Coccus | - | - | + | - |
| M28L2 | Yellow | Bacil | - | + | + | + |
| M30G1 | Orange | Coccus | - | - | + | - |
| M32G1 | Pink-Orange | Coccus | - | - | + | - |
| M33L1 | Yellow | Coccus | - | - | + | - |
| M33G2 | Creamy-white | Bacil | - | - | + | - |
| M37L1 | White | Coccus | + | - | + | - |
| M37L2 | White | Bacil | - | - | + | - |
| M39L1 | Creamy-white | Bacil | - | - | + | - |
| M41L1 | Yellow | Coccus | - | - | + | - |
| M41L3 | Yellow | Coccus | - | - | + | - |
| M44G1 | Yellow | Bacil | - | - | + | - |
| M45G3 | Yellow | Bacil | - | - | + | - |
| M46L1 | White | Bacil | - | + | + | - |
| M46L2 | Orange | Bacil | - | - | + | - |
| M46L4 | Yellow | Bacil | - | - | + | - |
| M46G1 | White | Coccus | - | - | + | - |
| M46G2 | Orange | Bacil | - | - | + | - |
| M46G3 | White | Coccus | + | - | + | - |
| M46G4 | Orange | Coccus | - | - | + | - |
| M49L1 | Pink-Orange | Bacil | - | - | + | - |
| M51G1 | Creamy-white | Bacil | - | + | + | - |
| M51G2 | Creamy-white | Bacil | - | + | + | - |
| M51G3 | Creamy-white | Coccus | - | - | + | - |
| M53L2 | White | Bacil | - | - | + | - |

**Extraction of Genomic DNA and Detection of *mxaF* Gene by PCR.** The genomic DNA of the isolates was extracted using CTAB method (Marchesi *et al*. 1998). Genomic DNA of the isolates was used as template in detection of *mxaF* gene. The expected amplicon size is approximately 550 bp. Most of the isolates show negative result of detection *mxaF* gene. Only one isolate from human feet (isolate K4-3) and one isolate from human mouth (isolate M28L2) were produced DNA fragment of the expected size (Figure 1).

**Enzyme Extraction and Enzyme Assay.** From the total 21 isolates obtained from the feet, 15 isolates showed MDH activity whereas 23 isolates from the total 37 isolates obtained from human mouth also showed MDH activity (Table 3 & 4). The highest total activity of methanol dehydrogenase comes from isolate K25-3 (74.444 U/ml), K33-6 (79.815 U/ml), and K43-5 (69.259 U/ml) from human feet and M41L3 (135.926 U/ml), M27G2 (85.556 U/ml), and M51G1 (103.333 U/ml) from human mouth.

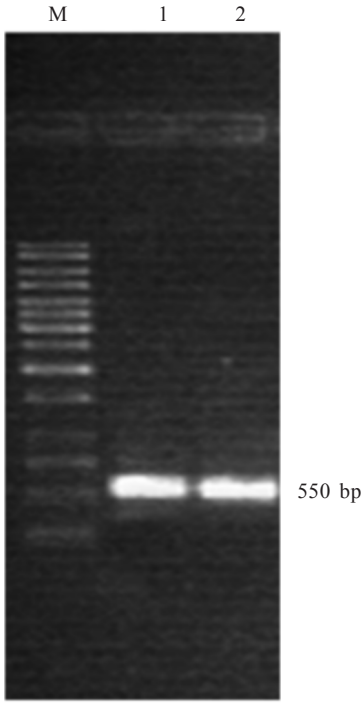


Figure 1. PCR detection of *mxaF* gene. M: Marker, 1: isolate K4-3, 2: isolate M28L2.

## DISCUSSION

Methanol dehydrogenase is an enzyme produced by methanol consuming bacteria such as methylotrophic

Table 3. The result of the activity assay of methanol dehydrogenase from human feet

| Isolates | Total activity (U/ml) | Protein concentration (mg/ml) | Specific activity (U/mg) |
|---|---|---|---|
| K4-2 | 18.120 | 0.013 | 1393.846 |
| K4-3 | 1.111 | 1.008 | 1.102 |
| K9 | 3.889 | 0.060 | 64.817 |
| K25-1 | 11.111 | 0.646 | 17.200 |
| K25-2 | 4.444 | 0.164 | 27.098 |
| K25-3 | 74.444 | 0.896 | 83.085 |
| K32-2 | 10.926 | 0.470 | 23.247 |
| K33-6 | 79.815 | 0.394 | 202.576 |
| K33-7 | 4.074 | 0.147 | 27.714 |
| K41-3 | 35.556 | 0.104 | 341.885 |
| K42 | 4.630 | 0.008 | 578.750 |
| K43-1 | 14.074 | 0.331 | 42.520 |
| K43-4 | 45.556 | 0.804 | 56.662 |
| K43-5 | 69.259 | 0.278 | 249.133 |
| K45-2 | 58.333 | 0.116 | 502.871 |

Table 4. The result of the activity assay of methanol dehydrogenase from human mouth

| Isolates | Total activity (U/ml) | Protein concentration (mg/ml) | Specific activity (U/mg) |
|---|---|---|---|
| M2G1 | 51.795 | 0.183 | 283.033 |
| M7L1 | 76.068 | 0.729 | 104.346 |
| M18G1 | 33.504 | 0.139 | 241.036 |
| M22L1 | 2.051 | 0.669 | 3.066 |
| M23L1 | 67.593 | 0.789 | 85.669 |
| M24G1 | 38.333 | 0.445 | 86.142 |
| M27G2 | 85.556 | 0.335 | 255.391 |
| M27L1 | 38.519 | 0.038 | 1013.658 |
| M27L3 | 56.410 | 0.032 | 1761.920 |
| M28L2 | 53.148 | 0.211 | 251.886 |
| M30G1 | 1.709 | 0.154 | 11.120 |
| M33G2 | 81.111 | 0.818 | 99.158 |
| M37L1 | 5.299 | 0.759 | 6.983 |
| M37L2 | 5.470 | 0.156 | 34.964 |
| M39L1 | 4.786 | 0.191 | 25.005 |
| M41L1 | 21.709 | 0.709 | 30.619 |
| M41L3 | 135.926 | 0.211 | 644.199 |
| M45G3 | 40.513 | 0.233 | 173.876 |
| M46G1 | 5.983 | 0.565 | 10.589 |
| M46L2 | 55.214 | 0.370 | 149.227 |
| M51G1 | 103.333 | 0.864 | 119.598 |
| M53L2 | 47.350 | 0.260 | 182.115 |
| M49L1 | 12.140 | 0.506 | 24.007 |

bacteria. This group of bacteria is known as one of microfloras in human feet and mouth. In this study, samples from human feet and mouth were collected and analyzed further.

The isolates had varied in pigmentation, Gram staining result, biochemical properties, and morphology (Table 1 & 2).

These results suggested the diversity of methylotrophic bacteria occurring in human feet and mouth. All isolates were catalase-positive, indicating that they have catalase enzyme which could degrade hydrogen peroxide to oxygen. Thus, it is suggested that all of the isolates were aerobic bacteria. This observation was similar to previous study which reported that biochemichal properties of methylotrophic bacteria isolated from human feet and mouth were catalase-positive (Anesti *et al.* 2004, 2005). It is suggested that catalase-positive is a characteristic of methylotrophic bacteria. Most of the isolates were Gram negative bacteria and oxidase-negative. This result showed that most of the isolates did not possess cytochrome oxidase which catalysed the transport of electrons from donor compounds to electron acceptors.

In order to detect the presence of *mxaF* gene, genomic DNA of the isolate was extracted and amplified by PCR using primers f1003 and r1561. These primers have previously been used to amplify *mxaF* gene from a variety of different environments (McDonald & Murrel 1997). Surprisingly, in this study PCR amplification results showed that most of the isolates did not possess the *mxaF* gene. Only one isolate from human feet (isolate K43) and one isolate from human mouth (isolate M28L2) produced the DNA fragments with the correct size (550 bp). In contrast, amplification of *mxaF* gene by Anesti *et al*. (2004 & 2005) showed that most of methylotrophic bacteria obtained from human feet and human mouth possess the *mxaF* gene. It may be possible that most of the isolates obtained in this study have different *mxaF* sequence. However, a total of 38 from 58 isolates showed the presence of methanol dehydrogenase activities (Table 3 & 4). These results supported the suggestion that the isolates obtained in this study may have different *mxaF* sequence.

The isolates were then further analyzed to determine MDH activity. After the crude extract of the enzymes were collected, the enzyme activity was determined based on spectrophotometry analysis (Table 3 & 4). From the total of 58 isolates obtained from both habitates, 38 isolates showed MDH activity. However, all isolates showed growth on minimal medium supplemented with 1% (v/v) methanol.

Methanol dehydrogenase activity was undetectable in other isolates presumably due to the the low production of the enzyme, the possible presence of interfering or inhibitory compounds in the crude extract and the reduced accuracy of the enzyme assay at low enzyme purity (Liu *et al.* 2006). Additionally, there might be certain factors that influenced the activity or the catalytic reaction of the enzyme, such as pH, temperature, and cofactors.

The specific activity might become higher than the enzyme activity since the specific activity is also depends on the protein concentration (Table 3 & 4). The specific activity presumably shows the purity of the enzyme. But due to the low purity, we cannot assume that the isolate with highest specific activity possesses the most potential enzyme. Therefore, the isolates with the highest total activity been picked instead of isolates with highest specific activity. These isolates were K25-3 (74.444 U/ml), K33-6 (79.815 U/ml), and K43-5 (69.259 U/ml) from human feet and M41L3 (135.926 U/ml), M27G2 (85.556 U/ml), and M51G1 (103.333 U/ml) from human mouth. However, the instability of methanol dehydrogenase activity might be found since the crude extract was used in enzyme assays. The methanol dehydrogenase from the isolate used by Liu *et al.* (2006) showed the stability of total activity after some steps of enzyme purification. Therefore, the purification of the methanol dehydrogenase could also

be considered due to the instability of the enzyme in the crude extract.

Further study on factors which affect the activity of the enzyme such as pH, temperature, cofactors is needed to get more information on characteristic of methanol dehydrogenase and increase the activation of methanol dehydrogenase.

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